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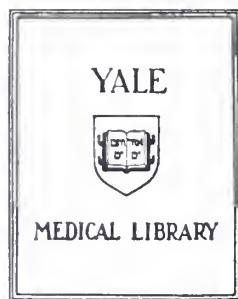


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ANTIBODIES OR αC DNA WHEN ADDED TO
SYSTEMIC LUPUS ERYTHEMATOSUS SERA

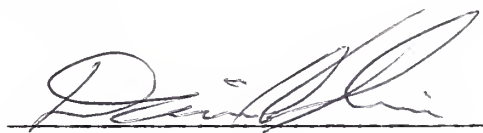
DAVID ALLEN SKRIER

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FIXATION OF COMPLEMENT BY ANTI-F(ab')₂ ANTIBODIES OR ssDNA
WHEN ADDED TO SYSTEMIC LUPUS ERYTHEMATOSUS SERA

A Thesis Submitted to the Yale University
School of Medicine in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Medicine

by
David Allen Shrier
1984

ABSTRACT

FIXATION OF COMPLEMENT BY ANTI-F(ab')₂ ANTIBODIES OR ssDNA WHEN ADDED TO SYSTEMIC LUPUS ERYTHEMATOSUS SERA

David Allen Shrier

1984

Recently, it has been demonstrated that anti-F(ab')₂ antibodies directed against the F(ab')₂ portions of immunoglobulins are present in the sera of patients with rheumatic diseases. The in vitro complement fixing activity of these anti-F(ab')₂ antibodies, when directed against F(ab')₂ portions of immunoglobulins, is unknown. In this study, a solid phase total hemolytic complement assay was used to quantitatively determine the % complement fixation by anti-F(ab')₂ antibodies or ssDNA when added to serum from patients with systemic lupus erythematosus (SLE). Both autologous and homologous anti-F(ab')₂ antibodies with demonstrated anti-anti-ssDNA activity fixed complement when added to SLE sera and normal human complement. Furthermore, comparable amounts of anti-F(ab')₂ antibodies with minimal anti-anti-ssDNA activity fixed zero or minimal amounts of complement when added to the same lupus sera. ssDNA, when measured by the same assay with the same sera, fixed greater amounts of complement than did comparable amounts of any of the anti-F(ab')₂ antibodies.

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To my parents
for their love and understanding.

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INTRODUCTION

The immune system

It is difficult to conceive of a mechanism more lethal than the human immune response. It possesses the ability to identify and respond rapidly to foreign elements with exquisite specificity and potent effector mechanisms. These events are the result of a complex network of signals and interactions by a number of different cells which together provide "homeostasis" (1). This network is provided with checks and balances via regulatory cells (helpers or suppressors) acting at both the T-T and T-B cell level. One of the functions of this regulatory system is to govern the specificity and magnitude of the antibody response to foreign proteins.

The immune system was originally conceived of as a defense mechanism against only infectious agents. However, at the turn of the century, Ehrlich coined the phrase "horror autotoxicus" to describe the idea that the body might rebel against itself. He postulated the existence of strict "internal regulating contrivances" to prevent this type of rebellion (2). Indeed, we now know that one of the consequences of a malfunction of such a regulatory system is autoimmune disease. Autoimmunity has been defined by F.M.

Burnet as "A condition in which structural or functional damage is produced by the action of immunologically competent cells or antibody (directed) against normal components of the body." (3).

The reality of such self-destructiveness first became apparent with the description of autoimmune hemolytic anemia and from multiple experiments demonstrating that proper immunization could induce animals to form autoantibodies. These autoantibodies were postulated by Burnet, in his clonal selection theory, to be products of "forbidden clones" that were potentially self-reactive and had somehow escaped destruction (4). However, as clinical and experimental evidence of autoantibodies and autoimmune phenomena accumulated it became clear that autoreactive cells were normally present in animals and humans (2, 5). Thus, far from being a forbidden event, self-recognition now appears to be a basic principle of the immune system.

In 1974 N.K. Jerne (6) first advanced the network theory of the immune system which proposes that immunologic control is mediated by a complex network of anti-idiotypic antibodies. These antibodies are directed against unique antigens (idiotypes) created by the individual combining sites of antibodies. Idiotypes are also present on antigen recognition molecules on the surface of B and T lymphocytes and, under experimental conditions, anti-idiotypic antibodies can either stimulate or suppress the function of

those lymphocytes bearing the appropriate idiotypic antigens. Additionally, it has been shown that recognition of the antigens of the major histocompatibility complex is also important in cooperation between lymphocytes or lymphocytes and macrophages. Thus, the immune system appears to be an intricate network of signals and receptors in constant communication and perpetual readjustment through negative and positive feedback mechanisms to self and non-self antigens. The concept of self-recognition has evolved from "never" (ie. forbidden clones) to "always with control" (2).

Autoimmunity

Autoimmune diseases, therefore, are most likely a result of defective immunoregulation. One widely accepted theory maintains that self-reactive B cells which are always present normally remain dormant because of suppressor T cell control or because helper T cells capable of recognizing autoantigens are absent or inactive. Loss of control with subsequent hyperactivity of B cells may result from a decreased number of suppressor T cells or a defect in suppressor cell function secondary to either a primary defect or to autoantibody directed against their cellular surface (1, 4, 8, 9, 10). Control may also be bypassed if foreign antigens and self antigens are linked together so that the T cell recognizing the foreign part of the antigen

can help the B cell recognizing the self part of the same antigen complex. Such a process may occur after a foreign antigen such as a drug adheres to a cell's surface or after viral antigens appear on the surface of a virus infected cell. The result would be production of antibody to self antigens on the cell surface. Similarly, formation of antibodies (rheumatoid factors) against other antibodies linked to foreign antigens in an immune complex or nonspecific stimulation of B cells by adjuvants might also bypass the helper T lymphocyte gap.

Other postulated mechanisms for deregulation include a genetic defect or viral infection specific for helper or suppressor T cells that would decrease the effectiveness of that cell leading to an imbalance in the system. Imbalance might also occur secondary to a genetic defect rendering B cells incapable of producing antibody upon proper stimulation. This might lead to overreaction in another part of the immune system in addition to failure of the effector function of the antibody.

Immunopathology and SLE

The concept that immunologic injury to specific organs or tissues might occur as a result of antigen-antibody reactions immunologically unrelated to the structures injured was first voiced by Von Pirquet as early as seventy years ago (11). In the course of studying serum sickness in

humans he postulated that the coexistence of foreign serum antigens and homologous antibodies in the circulation resulted in the formation of toxic compounds which were probably the cause of rather specific vascular, renal, cardiac, cutaneous and joint lesions developing in tissues without immunologic relationship to the injected antigen. It was not until almost fifty years later that it was firmly established that antigen-antibody complexes per se are pathogenetic agents capable of inducing a variety of injuries, ranging from acute through chronic inflammation to hyaline degeneration in particular anatomic sites.

This concept of immunologically induced tissue injury was first applied to systemic lupus erythematosus (SLE), particularly to lupus nephritis, in 1948 when M.M. Hargraves et. al. first noted the lupus erythematosus (LE) cell phenomenon in the bone marrow of patients with SLE (12). Approximately ten years later the immunologic nature of the LE cell phenomenon, observed in approximately 75% of lupus patients, was established when gamma globulin (7S IgG) with specificity for deoxyribonucleoprotein was identified as the reactive serum fraction involved. In the years that followed further serologic studies of SLE have revealed that the disease is characterized by a generalized excessive autoantibody production. These autoantibodies are postulated to be produced by excessive B cell activity arising from a defect in suppressor T cells. Though the

exact mechanism for this is unknown, it is most likely the result of anti-lymphocyte antibodies acting on discrete subsets of T cells rather than a primary cellular defect (1, 4). It is these autoantibodies that then go on to form immune complexes with self antigens and in this way participate in the pathogenesis of SLE.

Autoantibodies in SLE

The work of many investigators has delineated a great number of different autoantibodies that may appear in the serum of SLE patients (10, 12). Among those currently recognized are antibodies directed against nuclear constituents (double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), deoxyribonucleoprotein, ribonucleoprotein, Sm antigen, and carbohydrate antigens), cytoplasmic constituents (ribosomes, carbohydrate antigens, lipid antigens), gamma globulin, soluble clotting factors, platelets, red blood cells, lymphocytes and double or single-stranded RNA. Some of this antibody activity may be seen in other diseases whereas some is relatively specific for SLE. For example, anti-deoxyribonucleoprotein activity (the LE cell phenomenon) occurs in approximately 15% of patients with other rheumatic diseases. Anti-ssDNA is also found in patients with rheumatoid arthritis (RA), chronic active hepatitis, primary biliary cirrhosis, Sjogren's syndrome and drug-induced lupus-like syndromes. On the

other hand, high titers of anti-dsDNA are seen essentially only in SLE (found in 40% of SLE patients) although low concentrations are present in other rheumatic diseases (12-15).

Antibodies to dsDNA were among the first to be described in lupus patients. Aside from their usefulness in diagnosis, measurements of anti-dsDNA antibodies are useful in monitoring patients, because of the close correlation of increased titers with clinical exacerbation. SLE patients with these antibodies have a high incidence of clinical renal disease and serum hypocomplementemia. Studies of SLE patients dying with renal disease have shown increased concentrations of anti-dsDNA antibodies in their kidneys compared to serum. These data strongly suggest a pathologic role for dsDNA-anti-dsDNA immune complexes in the genesis of glomerulonephritis in these patients (13, 15).

Antibodies to ssDNA probably also play a major role in the immunopathogenesis of SLE. They are found in the great majority of SLE patients (70-75%) though they are non-specific. As with anti-dsDNA, anti-ssDNA antibodies have been eluted in increased concentrations from the kidneys (compared to serum) of some SLE patients dying with glomerulonephritis. There has also been described a group of patients with prominent cutaneous lesions and severe renal disease who have anti-ssDNA antibodies but have repeatedly failed to demonstrate anti-nuclear and anti-dsDNA

antibodies. These data suggest that immune complexes composed of ssDNA-anti-ssDNA also play a pathologic role in glomerulonephritis in SLE patients (13, 15).

Immune complexes and hypocomplementemia

We opened this discussion of the immunopathologic basis of SLE with the concept of immune complex mediated tissue injury. In the past ten to fifteen years a large number of methods for detecting immune complexes have been developed including Clq-binding assays, polyethylene glycol (PEG) precipitation assays and Raji cell binding assays. All of these show marked elevations of circulating immune complexes (CIC's) in SLE sera during acute episodes of the disease (12). In addition, immune complex deposition is closely correlated with histologic evidence of inflammatory lesions and dysfunction of renal glomeruli, renal tubules, blood vessels, synovial tissues, pleural and pericardial membranes, and alveolar septae of the lung. On the other hand, cytotoxic antibodies which directly interact with in situ membrane antigens (eg. anti-red blood cell antibodies) play a more limited role in the pathogenesis of SLE. The localization and potential cytotoxicity of circulating and/or locally formed antigen-antibody complexes is determined by multiple factors. The molecular size of the complex, the antigen-antibody ratio, the ability to fix complement, the avidity of the antibody component, the ionic

charge of the complex and the clearance of CIC's by the reticuloendothelial system have all been implicated as determinants of the pathogenicity of immune complexes (8). However, activation of the classical complement pathway appears to be a prerequisite for the expression of immune complex mediated tissue injury (8, 15). In fact, hypocomplementemia is one of the prominent humoral immunologic abnormalities in patients with active SLE (10, 16).

Hypocomplementemia may result from increased utilization (eg. as a result of immune complex formation), reduced synthesis or a combination of the two. In SLE the mechanism of increased utilization is indicated by a number of findings. These include significant depression of early complement components in hypocomplementemic SLE sera, identification of early complement components in renal lesions and the complement fixing ability of anti-DNA antibodies when added to DNA in vitro using a liquid phase total hemolytic complement assay (12, 17, 18). Studies have also revealed that fixation of complement occurs via both the classical and alternative pathways of complement activation, though the former is more actively utilized. Regardless of which pathway is activated it has been clearly demonstrated that the complement fixing activity of DNA-anti-DNA complexes is directly associated with the presence of active lupus nephritis (12, 15, 19, 20). There is also

evidence that a variety of other immune complex systems participate in complement fixation and disease activity (12). However, the precise mechanism by which complement proteins participate in the inflammatory response in SLE in vivo remains to be clarified (8).

Anti-idiotypic antibodies and the network theory

We mentioned earlier that, in 1974, N.K. Jerne first advanced the network theory of the immune system in which anti-idiotypic antibodies play a regulatory role (6). Specifically, he suggested that the normal immune response not only includes production of specific antibody (Ab1) to a given antigen but that the resulting antibody can itself act as an antigen for the induction of a second anti-idiotypic antibody (Ab2). Amino acids of the hypervariable regions of the Ab1 molecule associated with the antigen combining site and a portion of the surrounding framework region make up the antigen structure of the idiootype (7). These amino acid sequences are all contained within the $F(ab')_2$ portion of the immunoglobulin (2). The anti-idiotypic antibody (Ab2) reacts with this antigenic structure and is therefore referred to as anti- $F(ab')_2$ antibody. In turn, the antigenic structure of the Ab2 molecule can also serve as an antigen for the induction of anti-anti-idiotypic antibody (Ab3) (7).

The existence of such a regulatory network has been confirmed in animal models and in man by the work of many investigators (5, 21-23). We now know that this regulatory system is complicated and involves interactions between T and B cells bearing the target idiotypes or anti-idiotypes as well as interactions between those cells and circulating anti-idiotypic or anti-anti-idiotypic antibodies. In some systems, anti-idiotypes can suppress idiotypic expression whereas in others they have been shown to enhance expression of idiotypic (21-23). Of note is that although the above conclusions are based on experimental systems in which anti-idiotypic antibodies have been administered before or during the course of an immune response, there is also evidence that idiotypic regulation occurs during the normal immune response (21).

Complement fixing ability of anti-F(ab')₂ antibodies

It has recently been demonstrated that anti-F(ab')₂ (auto-anti-idiotypic) antibodies directed against the F(ab')₂ portions of immunoglobulins are present in the sera of patients with rheumatic diseases or IgA deficiencies as well as in normals (5, 24-27). These anti-F(ab')₂ antibodies in patients with SLE have been shown to include cross-reacting antibodies with specificity for anti-DNA (idiotypic) and are believed to have a role in the regulation of the immune response in accordance with Jerne's network

theory (5-6, 23, 25, 28). In vivo studies have suggested that, in lupus patients, high titers of anti-F(ab')₂ antibodies are associated with low titers of anti-DNA and low levels of disease activity (5). This has been taken one step further by Hahn and colleagues (28) who have demonstrated that, in NZB/NZW mice, survival was significantly prolonged in those mice treated with anti-idiotypic (anti-anti-DNA) and antibodies to DNA were transiently diminished in quantity versus those mice not similarly treated. However, it has also recently been demonstrated by Lambert and colleagues (29-30) that BALB/c mice immunized with lipopolysaccharide (LPS) or infected with trypanosomes exhibited idiotype-anti-idiotypic CIC's. This group then went on to show that these same immune complexes are deposited in glomerular lesions in BALB/c mice injected with LPS and may well be pathogenic (31).

As pointed out previously the complement activity of anti-DNA has been directly associated with the presence of active lupus nephritis in humans and anti-DNA antibodies are known to be complement fixing when added to DNA in vitro. The complement fixing activity of anti-F(ab')₂ antibodies in patients with SLE has not been studied, but has obvious clinical significance given the preceding considerations.

Thus, in this study we have tested the complement fixing activities of ssDNA and anti-F(ab')₂ antibodies when added to SLE sera with various anti-ssDNA antibody activities.

Using a solid phase sensitized sheep red blood cell total complement assay (Kallestad, Chaska, Minn.) we have shown that autologous and homologous anti-F(ab')₂ antibodies, with known anti-anti-ssDNA activity, fixed complement when added to SLE sera.

However, relatively greater quantities of complement were fixed when comparable amounts of ssDNA were added to the same sera. In both the ssDNA and anti-F(ab')₂ complement fixation assays there was a tendency for the higher ssDNA binding sera to fix greater amounts of complement than the lower ssDNA binding sera. Furthermore, anti-F(ab')₂ antibodies with minimal anti-anti-ssDNA activity fixed little or no complement when added to the same SLE sera.

MATERIALS AND METHODS

Patients and sera

Sera were obtained by venipuncture from informed patients at the UCLA Center for the Health Sciences and from normal volunteers. The patients with systemic lupus erythematosus (SLE) fulfilled the American Rheumatism Association criteria for classification of SLE (32) and the patients with rheumatoid arthritis (RA) fulfilled criteria for "Definite" or "Classical" RA (33). All samples were frozen at -20°C until use.

Single-stranded DNA was prepared by heating preparations of calf thymus DNA (Sigma Chem. Co., St. Louis, Mo.) at 100°C for 10 mins. and then rapidly cooling in an ice bath.

Immunoglobulin and its fragments

Human IgG was prepared from Cohn Fraction II (Miles Lab, Elkhart, Ind.) by elution from DEAE-cellulose column chromatography with 0.01 M potassium phosphate buffer, pH 8.0. F(ab')₂ fragments were prepared by pepsin digestion of IgG (24, 34). The F(ab')₂ was separated by collecting the excluded peak from a Sephadex G-75 column and subjecting this to two successive purifications using Sephadex G-200 to remove small amounts of undigested IgG, Fab and other small

fragments. This $F(ab')_2$ preparation was free of Fc fragments and undigested IgG by Ouchterlony analysis against specific rabbit anti-human Fc antiserum (Behring, Somerville, N.J.) at concentrations of up to 24 mg/ml and shown to have a purity greater than 98%. Concentrations of 1 gm/dl of this $F(ab')_2$ preparation bound 8% ssDNA in the Farr assay (see below).

Purification of specific antibodies by affinity column chromatography

Anti- $F(ab')_2$ antibodies were purified from the sera of patients with SLE and RA by affinity column chromatography using $F(ab')_2$ conjugated to Sepharose 4B (Pharmacia, Uppsala, Sweden). The immunoabsorbent contained approximately 5 mg of $F(ab')_2$ /ml of gel. Antibodies were eluted from the column by lowering the pH of the column to 3.5 in 0.1 M glycine, 1.5 M NaCl. These anti- $F(ab')_2$ antibody preparations were concentrated to 1/10th of the original serum volume by ultrafiltration, to a final protein concentration of approximately 300-600 ug/ml.

Anti-Fc antibodies (19S) were purified from the serum of an RA patient. The serum was first precipitated with 50% saturated ammonium sulfate. The precipitant was passed through two successive columns of IgG coupled to Sepharose 4B and the eluted IgM fraction was then purified by exclusion from a Sephadex G200 column. Any contaminating anti- $F(ab')_2$ antibodies were then removed from the anti-Fc

antibodies by exclusion chromatography from an $F(ab')_2$ Sepharose 4B column. The excluded effluents of IgM anti-Fc antibodies (19S) had anti-IgG titers of 80,000 or greater and negative anti- $F(ab')_2$ titers by radioimmunoassay (RIA) as described below. IgM (19S) anti-Fc antibodies (anti-IgG titers of 800) were used to enhance the reaction of anti- $F(ab')_2$ antibodies with ^{125}I -aggregated $F(ab')_2$ (35).

Radioimmunosassay for anti-IgG and anti- $F(ab')_2$ antibodies

The radioimmunoassay for anti-IgG and anti- $F(ab')_2$ was done in the manner previously described (24-25). IgG and $F(ab')_2$ fragments were radiolabeled with ^{125}I (Amersham Corp., Arlington Heights, Ill.) to a specific activity of 0.1 mCi/mg by the chloramine-T method (36). Aggregations were performed by heating 10 mg/ml of labeled proteins for 30 mins. at 63°C for IgG and 60°C for 20 mins. for $F(ab')_2$. Aggregates of IgG (A-IgG) and $F(ab')_2$ (A-Fab) with molecular weights of approximately 1×10^7 daltons were separated by sucrose density gradient (10-30%) ultracentrifugation at 180,000 g for 4 hrs. with a Beckman Sw40 rotor (Beckman, Palo Alto, Cal.) at 4°C (37).

Fifty microliters of purified anti- $F(ab')_2$ or anti-IgG antibodies were twofold serially diluted with 0.01 M sodium phosphate-buffered saline, pH 7.4 (PBS), containing 0.05% NaN_3 and 0.5% bovine serum albumin (BSA) in a microtiter plate (Dynatech., Alexandria, Va.). Then 100 ul of PBS

containing 0.5% BSA was added to each well and heat-inactivated at 56°C for 30 mins. Fifty microliters of each diluted sample were transferred to two different microtiter plates. A-IgG or A-Fab (0.025 ug in 25 ul) were added to each well, followed by 50 ul of 3% polyethylene glycol (3% PEG-6000), 0.02 M EDTA, and approximately 10,000 cpm of ^{22}Na (Amersham) as a volume marker. The wells containing A-Fab also received 25 ul of purified IgM-19S-anti-Fc antibodies while the wells containing A-IgG received 25 ul PBS. The plates were then agitated and incubated at room temperature for 16 hrs. The plates were centrifuged at 2000 g for 40 mins., the supernatants were aspirated and the pellets were counted for ^{125}I and ^{22}Na activities in a dual-channel automated gamma counter (Beckman). The titer of anti-IgG was defined as that dilution which precipitated 33% of the antigen whereas the percent binding for a given concentration of anti-F(ab')₂ was calculated as $[(^{125}\text{I} \text{ in precipitate}) / (^{125}\text{I} \text{ in } 25 \text{ ng of A-Fab})] \times 100\%$.

Radioimmunoassay for anti-ssDNA antibodies and the inhibition assays

Anti-ssDNA antibody activities were determined by a modified Farr test as described previously (38). Briefly, 10 ul of serum and 80 ul of borate saline containing 0.5% BSA and approximately 10,000 cpm of ^{22}Na , as a volume marker, were mixed and heat inactivated. Ten microliters of ^{125}I -ssDNA (0.034 ug) was added and incubated at 37°C and

4°C for 1 and 16 hrs., respectively. Then 100 ul of saturated ammonium sulfate was added and incubated at 4°C for 30 mins. and centrifuged at 1000 g for 30 mins. The supernatants were decanted and the pellets were counted for ^{125}I and ^{22}Na activities. The percentage DNA binding was calculated as $[(^{125}\text{I} \text{ in precipitate}) / (^{125}\text{I} \text{ in } 10 \text{ ul of } ^{125}\text{I}\text{-ssDNA})] \times 100\%$.

For the inhibition tests, the Farr test was slightly modified. Five microliters of serum and 80 ul of borate buffered saline containing 0.5% BSA and ^{22}Na were mixed and heat inactivated. Five microliters of inhibitor was added and incubated at 37°C and 4°C for 1 hr. each. Then 10 ul of ^{125}I -ssDNA was added, and the assay was carried out in the same manner as for anti-ssDNA antibodies. The percent inhibition was determined by $[((\% \text{ binding with PBS}) - (\% \text{ binding with inhibitor})) / (\% \text{ binding with PBS})] \times 100\%$.

Complement fixation

Levels of complement fixation were assayed using Kallestad Quantiplate total complement test kits (Kallestad, Chaska, Minn.). Each plate within a kit contained sheep red blood cells sensitized with hemolysin and standardized and immobilized in an agarose gel medium. The serum and/or mixture of reagents to be tested was placed in wells of equal dimensions and diffused radially through the agarose gel. The sheep red blood cells were lysed by complement to

form a cleared round zone. The total complement activity was measured by comparing the extent of lysis by the sample to that of reference sera using a semi-logarithmic graphical analysis.

Preparation of the test reagents involved first adding 25 ul of heat inactivated (56°C for 30 mins.) undiluted SLE serum to a test tube containing 25 ul of either ssDNA or purified anti-F(ab')₂ antibody. Next 25 ul of human complement (approximately 30 CH₁₀₀ units) obtained from normal (circulating immune complex and anti-ssDNA negative) human sera (as determined by a polyethylene glycol precipitation assay (38) and by the modified Farr test (38), respectively) was added to the test preparation. For control values, equal amounts of PBS were substituted for the appropriate reagent(s). After mixing, the preparation was incubated for 1 hour at 37°C. Then 5 ul of the test preparation, along with 5 ul of the appropriate reference sera and controls were placed in the Quantiplate Test Kit wells and incubated for 6 hrs. at 37°C. The diameters of the cleared zones were read with a Kallestad viewer and the levels of the total hemolytic complement (in CH₁₀₀ units) were determined as mentioned previously in accordance with Kallestad instructions.

RESULTS

Complement fixing ability of anti-F(ab')₂ antibodies in SLE sera

Anti-F(ab')₂ antibodies were purified by affinity column chromatography from SLE and RA patients' sera. Activities of the purified antibodies as measured by radioimmunoassay were shown to be positive (Table 1). Specificity of the purified anti-F(ab')₂ antibodies for anti-ssDNA antibody was determined by their ability to inhibit the ¹²⁵I-ssDNA reaction with the anti-ssDNA. In our previous report (25) we demonstrated that purified anti-F(ab')₂ antibodies from SLE sera inhibited the reactions between anti-ssDNA in sera with ¹²⁵I-ssDNA antigen; we also demonstrated that anti-F(ab')₂ antibodies purified from RA sera did not inhibit these same reactions. In this study we used two of the same purified anti-F(ab')₂ antibody preparations from the earlier study (RL and RI). In addition, we purified anti-F(ab')₂ antibodies from the serum of SLE patient DW. The percent inhibition of this purified anti-F(ab')₂ antibody (DW) as defined under Methods was measured to be 44% using serum from patient MH (59% ssDNA binding); the prior study indicated that SLE serum RL had a mean percent inhibition of 70% whereas RA serum RI had only 8% mean inhibition (25).

Thus, the anti-F(ab')₂ antibodies purified from SLE sera contain antibodies with greater specificity for anti-ssDNA antibodies than those anti-F(ab')₂ antibodies purified from the RA serum.

The ability of purified anti-F(ab')₂ antibodies to fix complement when added to SLE serum was determined using the solid phase total hemolytic complement assay as described under Methods. The results of this assay are presented in Figure 1. The amount of complement fixed is given in terms of percent complement fixed. This is defined as
$$\frac{[(\text{complement activity present in complement source} + \text{serum} + \text{PBS}) - (\text{complement activity present in complement source} + \text{serum} + \text{anti-F(ab')}_2)]}{(\text{complement activity present in complement source} + \text{serum} + \text{PBS})} \times 100\%$$
. The control serum (PC) was obtained from a normal human volunteer with a negative DNA binding test (< 20% binding). Also, controls were run in which high concentrations (≥ 300 ug/ml) of anti-F(ab')₂ antibodies purified from SLE or RA serum were added to the complement source without the addition of SLE serum. In none of these control runs was more than 15% of the total complement activity fixed.

The anti-F(ab')₂ antibody purified from SLE serum fixed complement when combined with either autologous (DW) or homologous (AS, MH, DH, BJ, PS) SLE serum (Figure 1A). Maximum complement fixation occurs at high concentrations of anti-F(ab')₂ antibodies and decreases or remains steady as the concentration of anti-F(ab')₂ antibody decreases.

TABLE 1

Purified Anti-F(ab')₂ Antibody Activities as Measured by
Percent Binding of 25 ng A-Fab

| | Concentration of Anti-F(ab') ₂ Antibodies (ug/ml) (after purification & concentration) | Percent Binding of 25 ng A-Fab* |
|--|--|------------------------------------|
| <hr/> | | |
| Anti-F(ab') ₂ from <u>SLE Patients</u> | | |
| RL | 200 | 90 |
| DW | 500 | 61 |
| | | |
| Anti-F(ab') ₂ from <u>RA Patient</u> | | |
| RI | 30 | 33 |

Note: RL and DW represent individual SLE patient donors.
RI represents an individual RA patient donor.

* These values can not be used for comparison since the concentrations of anti-F(ab')₂ antibodies differ greatly. The values are provided only to indicate that the anti-F(ab')₂ antibodies are indeed active.

FIGURE 1

A The percent complement fixation of various SLE sera or a normal control serum when added to various concentrations of anti-F(ab')₂ antibody purified from SLE serum DW.

a....Figures in parentheses indicate anti-ssDNA antibody activity (% binding to 34 ng of ¹²⁵I-ssDNA) of the various sera.

b....Serum DW is autologous to the anti-F(ab')₂ antibody.

B The percent complement fixation of the same SLE sera or a normal control serum when added to 300 ug/ml of anti-F(ab')₂ antibody purified from RA serum RI.

c....Sera PS, BJ, DW, and PC fixed no additional complement when added to 300 ug/ml of RA anti-F(ab')₂ antibody.

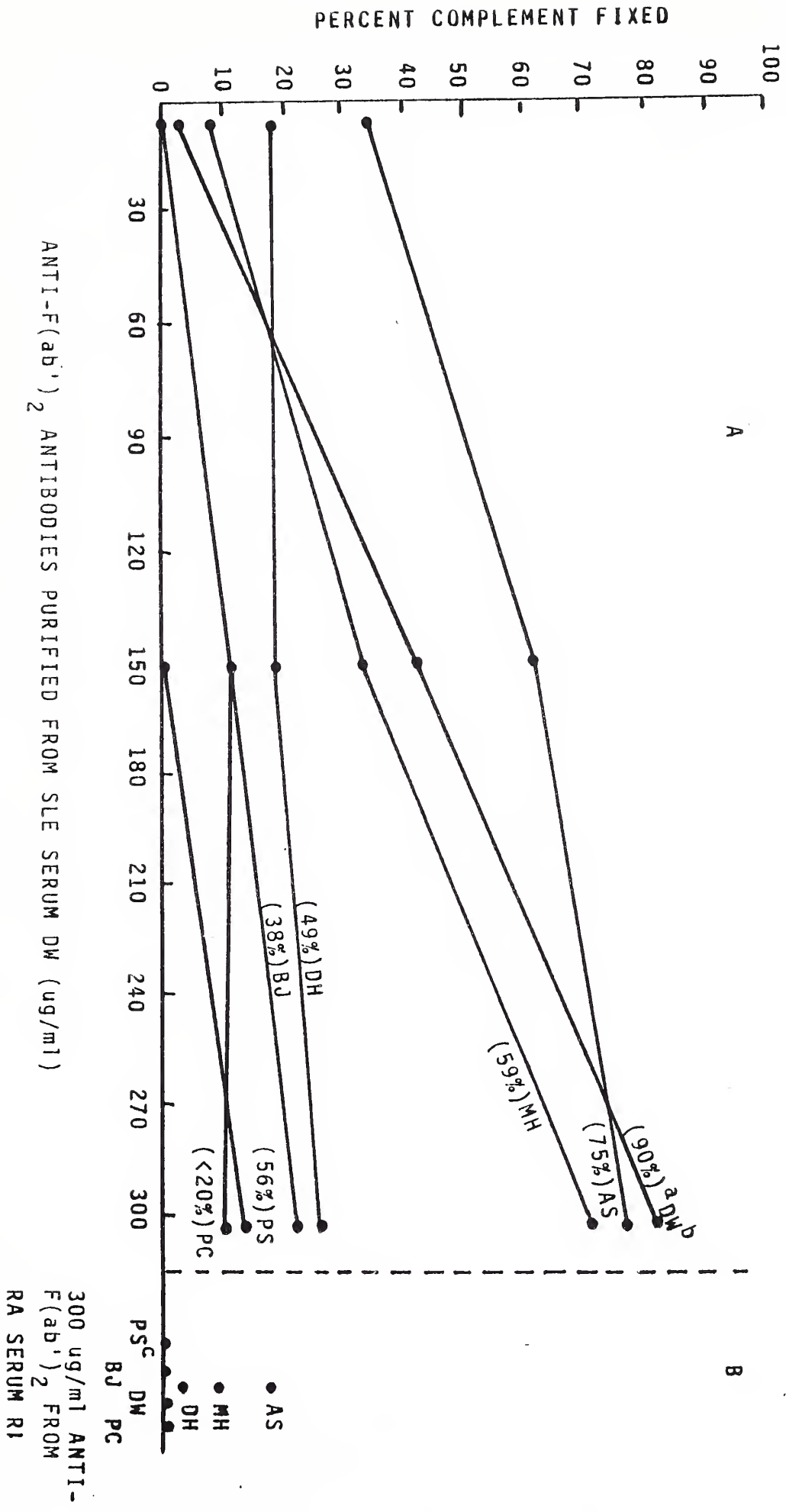
Note: DW, AS, MH, DH, BJ, and PS represent individual SLE patient donors.

RI represents an RA patient donor.

PC represents a normal donor.

Zero percent complement fixed is equivalent to the amount of complement fixed by serum alone.

FIGURE 1



Further, there is a tendency for the sera with higher anti-ssDNA activities to fix greater amounts of complement with anti-F(ab')₂ antibodies than did the sera with lower anti-ssDNA activities. The same SLE sera, (Figure 1B) when added to 300 ug/ml of anti-F(ab')₂ antibody purified from RA serum fixed significantly smaller amounts of complement ($p < 0.01$ by Student's T-test) than was fixed with the same concentration of anti-F(ab')₂ antibodies purified from SLE serum (Figure 1A). Thus, the purified anti-F(ab')₂ antibodies with anti-anti-ssDNA fixed significantly greater amounts of complement when combined with SLE sera than did purified anti-F(ab')₂ antibodies with no anti-anti-ssDNA activity.

Complement fixing ability of ssDNA antigen in SLE sera

The ability of ssDNA antigen to fix complement when added to SLE sera with various anti-ssDNA activities was determined in the same manner as was the complement fixing ability of the purified anti-F(ab')₂ antibodies discussed previously. The results are presented in Figure 2. Here, the term percent complement fixed is defined as

$$\left[\frac{(\text{complement activity present in complement source} + \text{serum} + \text{PBS}) - (\text{complement activity present in complement source} + \text{serum} + \text{ssDNA})}{(\text{complement activity present in complement source} + \text{serum} + \text{PBS})} \right] \times 100\%$$

The control serum used was the same as that used for Figure 1. Also, controls were run

in which high concentrations of ssDNA (≥ 21 ug/ml) were added to the complement source without the addition of SLE serum. In none of these control runs was more than 10% of the total complement activity fixed.

As shown in Figure 2, ssDNA fixed complement when combined with SLE sera previously shown to have various anti-ssDNA activities. There was a tendency for the sera with higher anti-ssDNA activities to fix greater amounts of complement with ssDNA than did the sera with lower anti-ssDNA activities. A comparison of Figures 1A and 2 shows that ssDNA (5.5 ug/ml), when combined with SLE sera, fixed significantly greater amounts of complement ($p < 0.05$ by Student's T-test) than did comparable amounts (6.25 ug/ml) of purified SLE anti-F(ab')₂ antibody when combined with the same SLE sera.

FIGURE 2

The percent complement fixation of various SLE sera or a normal control serum when added to various concentrations of ssDNA.

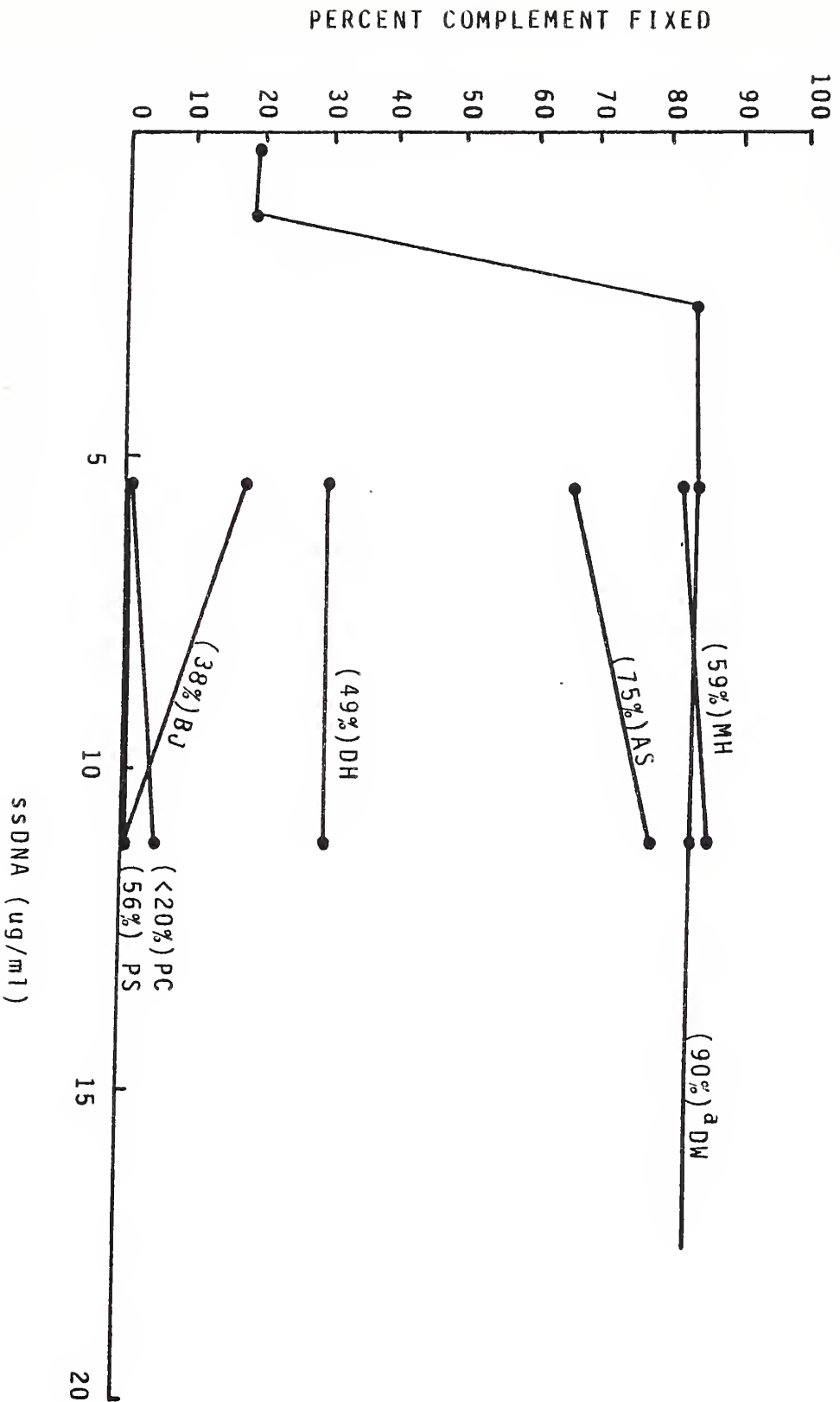
a....Figures in parentheses indicate anti-ssDNA antibody activity (% binding to 34 ng of ^{125}I -ssDNA) of the various sera.

Note: DW, AS, MH, DH, BJ, and PS represent individual SLE patient donors.

PC represents a normal donor.

Zero percent complement fixed is equivalent to the amount of complement fixed by serum alone.

FIGURE 2



DISCUSSION

It has recently been demonstrated that anti-F(ab')₂ (anti-idiotypic) antibodies are present in the serum of patients with certain autoimmune diseases and in normal individuals (5, 24-27). Furthermore it has been demonstrated that such anti-idiotypic antibodies may participate in in vivo immune complex formation (5, 29-31). In this study, we have shown that purified anti-F(ab')₂ antibodies with anti-anti-ssDNA activity fixed complement when combined with autologous or homologous SLE sera (Figure 1A). Comparable amounts of anti-F(ab')₂ antibodies with no anti-anti-ssDNA activity fixed significantly smaller amounts of complement when combined with the same SLE sera (Figure 1B). Furthermore, ssDNA, when added to the same SLE sera, fixed significantly greater amounts of complement than did comparable amounts of purified anti-F(ab')₂ with anti-anti-ssDNA activities (Figures 1A and 2).

We have previously pointed out that there was a tendency for higher ssDNA binding SLE sera to fix greater amounts of complement than lower ssDNA binding sera, when added to either SLE anti-F(ab')₂ or ssDNA. It is evident, from Figures 1A and 2, that serum from patient donor PS did not show this. Patient donor PS was the only patient positive

for anti-ssDNA, anti-ribonucleoprotein (anti-RNP) antibodies and normal C3 levels. Previous investigations (15) have demonstrated that the presence of anti-RNP antibodies in patients with SLE is associated with milder forms of nephritis. Thus, we may speculate, that anti-RNP, via some unknown mechanism, may interfere with DNA binding and/or complement fixation activity.

A second possibility for the seemingly aberrant behavior of serum PS may involve normal variations of the complement fixing ability of anti-DNA antibodies not directly related to the absolute quantity of anti-DNA present (19).

Such heterogeneity in the complement fixing activity of anti-ssDNA may reflect variations in IgG subclass concentrations of this antibody from serum to serum. Likewise, heterogeneity in the complement fixing activity of anti-F(ab')₂ antibodies may reflect variations in the relative concentrations of "private" or autologous anti-F(ab')₂ and cross-reactive or homologous anti-F(ab')₂ antibodies. That is, the differences in the complement fixing activities of the anti-F(ab')₂ antibodies may be attributable to the concentrations, avidities and cross-reactivities of the antibodies in the original sera which were absorbed and then eluted from the relatively small amounts of anti-ssDNA antibodies in normal human F(ab')₂ used for purification. We have obtained some further data which suggests these possibilities. Figure 3 demonstrates

that SLE serum DW, when added to autologous anti-F(ab')₂ antibodies, fixed substantially greater amounts of complement than the same serum added to homologous SLE anti-F(ab')₂ antibodies. It has been demonstrated that there is normal variation in the complement fixing activity of anti-DNA antibodies when added to DNA (19). Our data suggests that there is also normal variation in the complement fixing activity of anti-ssDNA antibodies when added to anti-F(ab')₂ antibodies with anti-anti-ssDNA activity.

These observations may provide insight into the pathogenesis of lupus nephritis. It is well known that the complement fixing activity of circulating immune complexes is associated with hypocomplementemia and tissue destruction (2, 8, 10, 19, 39, 40). Therefore, it is possible that the interaction of idiotype with anti-idiotype occurring within immune complexes simply fixes complement in addition to that fixed by the binding of antigen with antibody. This may directly result in increased tissue destruction by complement and represent the mechanism by which idiotypic interactions could be involved in the pathogenesis of glomerular disease as suggested by Goldman and colleagues (30-31). On the other hand, complement may solubilize the immune complexes (41-43) and perhaps alter their pathogenicity (44). Preliminary data from our laboratory has suggested, via a polyethylene glycol (PEG) precipitation assay (38) that addition of anti-F(ab')₂ antibodies to SLE

FIGURE 3

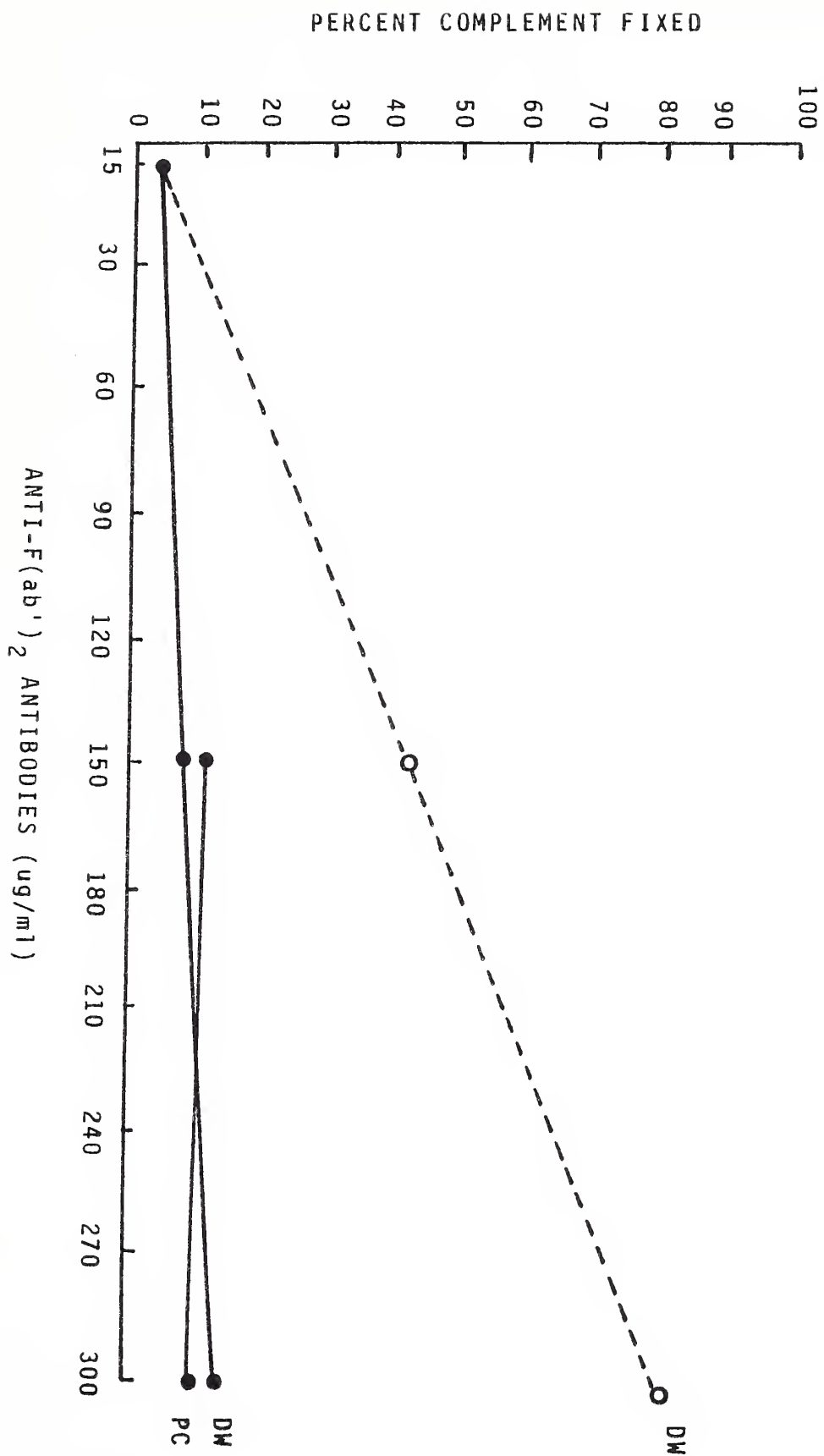
The percent complement fixation of SLE serum DW or normal control serum PC when added to various concentrations of autologous¹ (O--O) or homologous² (●--●) purified anti-F(ab')₂ antibodies.

¹....Purified from SLE serum from patient donor DW.

²....Purified from SLE serum from patient donor RL.

Note: Zero percent complement fixed is equivalent to the amount of complement fixed by serum alone.

FIGURE 3



sera with complement present may reduce the size of immune complexes. What effect this might have on the pathogenicity of these immune complexes requires future clarification. An additional possibility for the role of anti-idiotypic antibodies may involve an ability to block antigen-antibody binding (25) without fixing complement. That is, perhaps concentrations of anti-idiotypic which are too low to fix complement with idiotype (antibody) can yet interfere with antigen-antibody binding. This concept is in accordance with Jerne's network theory of auto-regulation by anti-idiotypic antibodies (6) and with the experimental work provided by many investigators including Abdou et. al. (5) and Hahn and colleagues (23, 28). It remains to be seen precisely in what way complement fixation by anti-idiotypic antibodies plays a role in immunoregulation via the network system of antibodies and lymphocytes.

In this study we have provided evidence that purified anti-F(ab')₂ antibodies with anti-anti-ssDNA activity fix complement when added to SLE sera with anti-ssDNA binding activity. A logical extension of this work would involve adding anti-F(ab')₂ antibodies together with ssDNA to SLE sera. Such a system would test the ability of anti-F(ab')₂ antibodies to inhibit or augment complement fixation by ssDNA when added to SLE sera. One could speculate that, by blocking antigen-antibody binding, anti-F(ab')₂ antibodies may in fact reduce complement fixation by ssDNA-anti-ssDNA

immune complexes. It would also be extremely valuable to run the complement fixation assays with purified anti-ssDNA and anti-anti-ssDNA antibodies. This would eliminate the influence of any possible cross-reacting idiotypes and provide further evidence that the complement fixation is indeed specifically related to the binding of anti-F(ab')₂ antibodies to anti-ssDNA. At the time the current research was carried out purified antibodies were available but in insufficient quantity. Such a problem could easily be overcome in the future by careful planning. Other possibilities for future work include obtaining further data on the variations in size of the immune complexes with complement fixation and a comparison study of the complement fixing ability of anti-idiotypic with idiotype versus the ability of anti-idiotypic to block antigen-antibody binding. An inverse correlation in the latter study may have positive implications for any possible therapeutic role for anti-idiotypic antibodies (23).

Thus, for the findings in this study to be clinically significant, further investigations into the nature of the complement fixing ability of antigen, antibody and auto-anti-idiotypic antibody complexes are needed.

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